# REMOVAL OF PHOSPHATE GROUPS FROM CASEIN WITH POTATO ACID PHOSPHATASE

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## Summary

Potato acid phosphatase (EC 3.1.3.2) was used to remove the eight phosphate groups from $\alpha_{s1}$-casein. Unlike most acid phosphatases, which are active at pH 6.0 or below, potato acid phosphatase can catalyze the dephosphorylation of $\alpha_{s1}$-casein at pH 7.0. Although phosphate inhibition is considerable ($K_I$ = 0.42 mM phosphate), the phosphate ions produced by the dephosphorylation of casein can be removed by dialysis, allowing the reaction to go to completion. The dephosphorylated $\alpha_{s1}$-casein is homogeneous on gel electrophoresis with a slower mobility than native $\alpha_{s1}$-casein and has an amino acid composition which is identical to native $\alpha_{s1}$-casein. Thus the removal of phosphate groups from casein does not alter its primary structure. Potato acid phosphatase also removed the phosphate groups from other phosphoproteins, such as, $\beta$-casein, riboflavin binding protein, pepsinogen, ovalbumin, and phosvitin.

## Introduction

Although dephosphorylated casein has been prepared using spleen phosphoprotein phosphatase [1,2], the procedure has been unsatisfactory in several respects. Examination of the properties of several phosphatases indicated that potato acid phosphatase (EC 3.1.3.2) might have certain advantages over spleen phosphoprotein phosphatase for removing the phosphate groups from casein. Since potato acid phosphatase is active at pH 7.0, the precipitation of dephosphorylated casein which occurs at pH 6.0 using spleen phosphoprotein phosphatase is avoided. Potato acid phosphatase requires no activators, while spleen phosphoprotein phosphatase requires reducing conditions for maximum activity. When reducing agents, such as $\beta$-mercaptoethanol, are used with the spleen enzyme, proteins having disulfide bonds are denatured while being dephosphor-

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ylated. Potato acid phosphatase also has the advantage of being available commercially in a highly purified form.

Potato acid phosphatase is a typical acid phosphatase, catalyzing the hydrolysis of a large variety of phosphoric acid mono-esters, but not the hydrolysis of di- and tri-esters, phosphoamides or ATP [3]. Although its kinetic behavior has been well characterized using substrates such as phenyl phosphate [4], *p*-nitrophenyl phosphate [5,6], and β-glycerophosphate [5,6], less is known about its action on phosphoproteins. Previous studies have shown that potato acid phosphatase can be used to prepare dephosphorylated pepsin [7,8] and partially dephosphorylated whole casein [9]. Since pure casein fractions are now available, it seemed worthwhile to reexamine the dephosphorylation of casein with potato acid phosphatase. Our main objective has been to prepare homogeneous dephosphorylated $\alpha_{s1}$-casein for use as a substrate for mammary gland casein kinase [10]. The procedure which we have developed is simple and should be useful to investigators interested in removing the phosphate groups from casein as well as other phosphoproteins under relatively mild conditions.

## Materials and Methods

### *Materials*

Milk proteins were isolated from cow's milk of known genetic composition. $\alpha_{s1}$-Casein B and β-casein $A^2$ were prepared by the method of Aschaffenburg [11] and further purified on DEAE-cellulose [12]. The phosphopeptide from β-casein, prepared by the method of Peterson et al. [13], was a gift from Dr. R.F. Peterson of this laboratory. Potato acid phosphatase, Grade A (activity: 160 I.U./ml at 25° C), was obtained from Calbiochem * and was diluted with 1% bovine serum albumin before use. Riboflavin binding protein was purified from egg white [14]. Pepsinogen was a product of Worthington Biochemical Corporation. Ovalbumin was obtained from Sigma Chemical Company and phosvitin was obtained from Nutritional Biochemical Corporation. *p*-Nitrophenyl phosphate, dicyclohexylammonium salt, was a product of Calbiochem and DL-*O*-phosphoserine was a product of Mann Research Laboratories.

### *Phosphatase assay*

The activity of potato acid phosphatase was measured in a 1 ml reaction mixture containing 100 μmol acetate buffer (pH 5.5), 5 μmol *p*-nitrophenyl phosphate, and 0.3 μg potato acid phosphatase. After incubation for 5 min at 38° C, the reaction was terminated by the addition of 3 ml 0.25 M NaOH. The released *p*-nitrophenol was determined by measuring the absorbance at 410 nm. Appropriate blanks were subtracted from the observed values. A unit of nitrophenyl phosphatase activity is defined as the amount of enzyme which releases 1 nmol of *p*-nitrophenol per min. The enzyme activity was linear with respect to enzyme concentration and incubation time.

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*Phosphoprotein phosphatase activity*

The activity of potato acid phosphatase on casein was measured in a 1 ml reaction mixture containing 5 mg of casein (1.7 μmol in phosphate equivalent), 100 μmol TES (*N*-Tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid) buffer (pH 7.0), and 1.0 μg enzyme. After incubation for 10 min at 38° C, the reaction was stopped by the addition of 0.4 ml of 0.02 M silicotungstic acid and analyzed for inorganic phosphate. A unit of activity is defined as the amount of enzyme which catalyzes the release of 1 nmol of inorganic phosphate per min. The activity was linear with respect to enzyme concentration and incubation time.

*Preparation and analysis of dephosphorylated casein*

$\alpha_{s1}$-Casein was treated with potato acid phosphatase in a 1 ml reaction mixture containing 2.5 mg of casein and 2 μg enzyme at pH 7.0. After incubation for 2 h at 38° C, the solution was frozen in a solid $CO_2$/acetone bath and freeze-dried. The preparation was then subjected to electrophoresis on polyacrylamide gel to determine the extent of dephosphorylation.

*Zonal electrophoresis*

Electrophoresis was carried out in an E-C vertical cell, using 7% polyacrylamide gel in 4.5 M urea with Tris buffer (pH 9.2) by the method of Thompson et al. [15]. A maximum voltage of 350 V at 80 mA was applied. Proteins on the gel were stained with 0.1% amido black in 7% acetic acid for about 30 min followed by electrolytic destaining of the background.

*Amino acid analyses*

Amino acid composition was determined by the automated procedure of Moore and Stein [16]. Phenol was added to protein samples before acid hydrolysis to minimize tyrosine destruction [17]. Analyses were made on samples hydrolyzed for 24 h at 110° C.

*Phosphate determination*

Inorganic phosphorus was determined by the method of Martin and Doty [18] and total phosphorus by the method of Meun and Smith [19].

*Protein determination*

Protein concentration was calculated from the absorbance at 280 nm using $E^{1\%}_{1cm}$= 15.0, 7.5, 10.2, and 4.6 for riboflavin binding protein (Farrell, H.M., Jr., unpublished observations), ovalbumin [20], $\alpha_{s1}$-casein [21] and β-casein [22], respectively. The concentration of pepsinogen was determined from the absorbance at 278 nm using $E^{1\%}_{1cm}$= 8.0 [23]. The concentration of phosvitin was estimated from its total nitrogen content, using the micro-Kjeldahl method [24]. The nitrogen content of phosvitin is 12.4% [25].

*High-voltage paper electrophoresis*

Electrophoresis was carried out for 3 h at 3.5 kV on Whatman No. 3MM paper using a pyridine-acetate buffer at pH 6.4 [26].

*pH optimum*

The effect of pH on potato acid phosphatase activity was determined on *p*-nitrophenyl phosphate and on casein (Fig. 1). The enzyme has a broad pH optimum with both substrates. The optimum pH for the *p*-nitrophenyl phosphatase activity is between 5.0 and 6.0, which is similar to the values reported by Alvarez [5]. Using $\alpha_{s1}$-casein as a substrate, the phosphoprotein phosphatase activity has an optimum pH between 5.8 and 7.0 retaining more than 90% of its maximum activity over this range. The *p*-nitrophenyl phosphatase activity is approximately 8 times higher than the phosphoprotein phosphatase activity when both are assayed at their pH optima.

*Kinetic data*

The effect of varying the casein concentration on phosphatase activity is shown in Fig. 2. The double reciprocal plot (Fig. 2, inset) was used to compute the apparent $K_m$. The $K_m$ value for $\alpha_{s1}$-casein at pH 7.0 is 2.2 mg per ml (0.75 mM in phosphate equivalents).

Double reciprocal plots in the presence and absence of orthophosphate were obtained using *p*-nitrophenyl phosphate as the substrate (Fig. 3). The results indicate that orthophosphate is a competitive inhibitor of potato acid phosphatase activity. At a *p*-nitrophenyl phosphate concentration of 5 mM, inhibition is evident with phosphate concentrations of 0.2 mM and 0.5 mM. A replot of the slopes vs. phosphate concentration is linear and the inhibition constant determined from the horizontal intercept of the replot gave a value of 0.42 mM

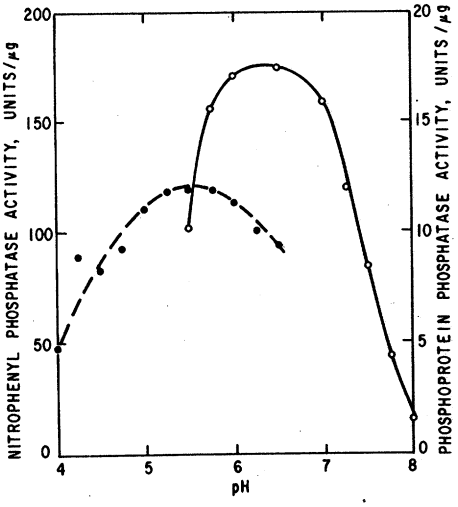


**Fig. 1. Effect of pH on phosphatase activity. *p*-Nitrophenyl phosphatase activity (●- - - -●) was measured in 0.1 M acetate buffer from pH 4 to 5.5 and in 0.1 M citrate buffer from pH 5.75 to 6.5. Phosphoprotein phosphatase (○———○) was measured in 0.1 M MES (2(*N*-morpholino)ethane-sulfonic acid) buffer (pH 5.5 —6.5) and in 0.1 M TES buffer (pH 7.0 to 8.5). Except for the buffers used, incubation conditions are as described under Materials and Methods.**

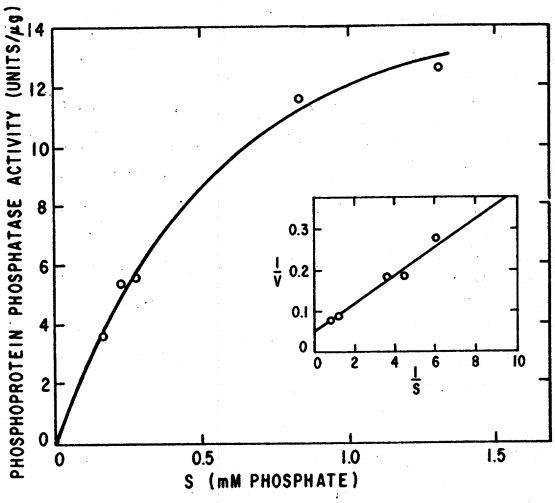


Fig. 2. Effect of the concentration of substrate on phosphoprotein phosphatase activity. The activity was assayed by the method described under Materials and Methods, except that the concentration of $\alpha_{s1}$-casein was varied. Inset shows a double reciprocal plot of the data. The line was drawn from a least squares analysis of the data, using the weighting methods of Wilkinson [37].

$KH_2PO_4$ (Fig. 3, inset). Inhibition data was not obtained for casein because of the difficulty of measuring phosphate released from casein in the presence of $KH_2PO_4$.

The kinetic constants are summarized in Table I. The $V$ for $\alpha_{s1}$-casein is six- to seven-fold lower than the $V$ for $p$-nitrophenyl phosphate, while the $K_m$ val-

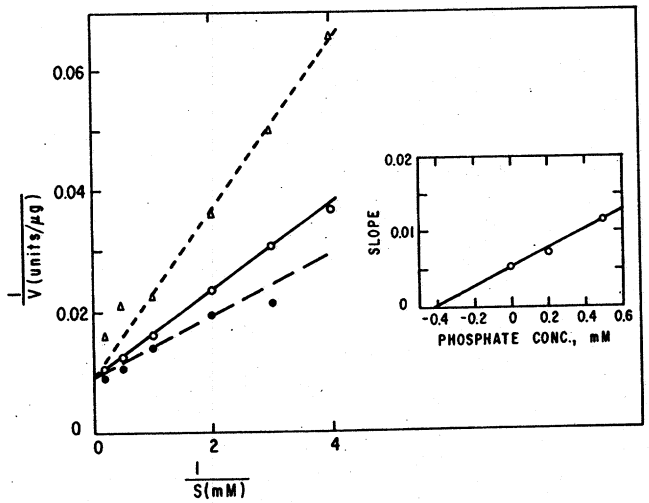


Fig. 3. Double reciprocal plots of the inhibition of $p$-nitrophenyl phosphatase activity by orthophosphate. The activity was measured as described under Materials and Methods, except that the concentration of $p$-nitrophenyl phosphate was varied. Lines were drawn according to the method described in Fig. 2. $K_2HPO_4$ concentrations were (●), no phosphate; (○), 0.2 mM and (△), 0.5 mM. The inset is a replot of the data, showing slope vs. phosphate concentration.

TABLE I

KINETIC CONSTANTS FOR POTATO ACID PHOSPHATASE

| | Substrates | |
|---|---|---|
| | Nitrophenyl phosphate | Casein |
| $V$ (nmol $min^{-1}$ $\mu g^{-1}$) | 120 | 18.9 |
| $K_m$ (mM in phosphate) | 0.62 | 0.75 |
| $K_i$ (mM in phosphate) | 0.42 | |

ues for the two substrates are similar. The $K_m$ for $\alpha_{s1}$-casein (0.75 mM) was calculated on the basis of 8 available phosphate groups and is equivalent to 0.093 mM $\alpha_{s1}$-casein. The $K_m$ of 0.62 mM for *p*-nitrophenyl phosphate agrees satisfactorily with the results of Alvarez [5], who reported a value of 0.83 mM.

*Substrate specificity*

Since the phosphate groups of casein are present as serine monoesters, the activity of potato acid phosphatase toward phosphoserine and a phosphopeptide from β-casein was investigated and the results are summarized in Table II. Both phosphoserine and phosphopeptide were hydrolyzed at a slower rate than casein at pH 7.0 while at pH 5.5 phosphoserine was hydrolyzed three times as fast as casein. The *p*-nitrophenyl phosphatase activity is eight times higher than the phosphoprotein phosphatase activity when both are assayed at their pH optima. Potato acid phosphatase also catalyzed the dephosphorylation of phosvitin and β-casein (Table II).

*Effect of incubation time on the dephosphorylation of casein*

$\alpha_{s1}$-Casein has 8 phosphate groups [28] and is homogeneous on acrylamide gel electrophoresis (pH 9.2). When $\alpha_{s1}$-casein is partially dephosphorylated, multiple bands appear on electrophoresis, which seem to represent $\alpha_{s1}$-casein

TABLE II

SPECIFICITY OF POTATO ACID PHOSPHATASE

Test system: 100 μmol TES buffer pH 7.0 or acetate buffer pH 5.5, 0.3—1.0 μg potato acid phosphatase and substrate as indicated in a final volume of 1 ml incubated at 38°C for 5 min. Phosphate release was measured as described under Materials and Methods. The *p*-nitrophenyl phosphatase activity was measured by the appearance of *p*-nitrophenol.

| Substrate | Amount of substrate per ml | Relative activity/μg potato acid phosphatase | |
|---|---|---|---|
| | | pH 5.5 | pH 7.0 |
| $\alpha_{s1}$-Casein | 5 mg | 75 | 100 |
| β-Casein | 5 mg | | 115 |
| Phosvitin | 5 mg | | 46 |
| β-Casein phosphopeptide | 1 mg | 58 | 54 |
| Phosphoserine (DL) | 5 μmol | 223 | 72 |
| *p*-Nitrophenyl phosphate | 5 μmol | 800 | 550 |

with varying amounts of phosphate [2]. When the phosphate groups of $\alpha_{s1}$-casein are completely removed, the dephosphorylated $\alpha_{s1}$-casein is homogeneous on acrylamide gel electrophoresis and has a slower mobility than native $\alpha_{s1}$-casein. Therefore acrylamide gel electrophoresis was used to monitor the reaction.

The effect of incubation time on the dephosphorylation of $\alpha_{s1}$-casein by potato acid phosphatase is shown in Fig. 4a. Although 60% of the phosphate is hydrolyzed in 1.5 h, little additional phosphate is released by longer incubation times. Examination of the electrophoretic patterns of the $\alpha_{s1}$-casein from this experiment confirms these results. Considerable changes in electrophoretic patterns occur in 1.5 h, but only minor changes occur for the next 3 h.

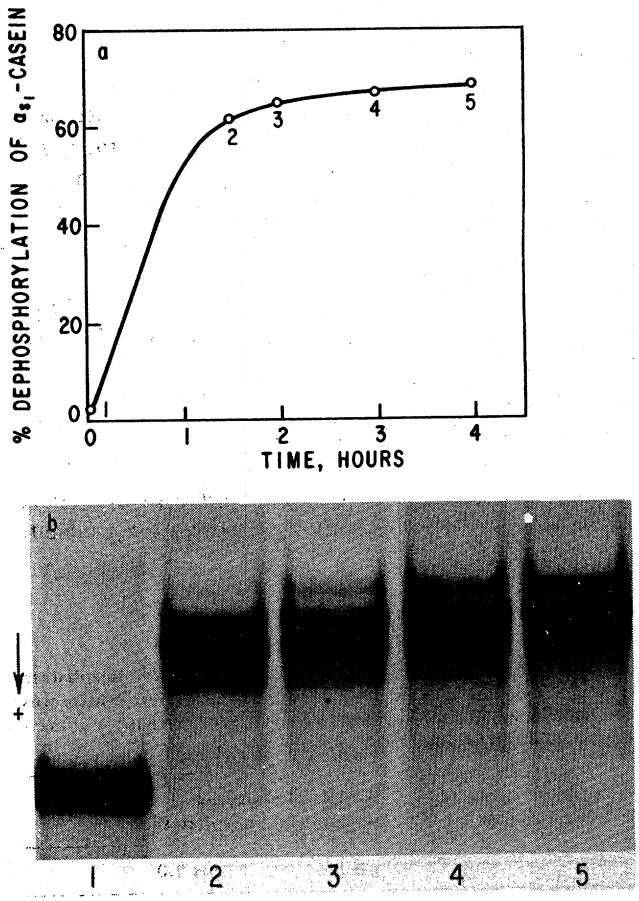


Fig. 4. (a) Phosphate released from $\alpha_{s1}$-casein by potato acid phosphatase as a function of incubation time. The reaction mixture (10 ml) contained 25 mg $\alpha_{s1}$-casein and 20 $\mu$g potato acid phosphatase at pH 7.0 and 38°C. Aliquots were removed at the indicated times and were analyzed for inorganic phosphorus by the method of Martin and Doty [18]. A second aliquot was freeze-dried and subjected to electrophoresis as shown in (b). (b) Polyacrylamide gel electrophoretograms of the five fractions as indicated in (a). Electrophoresis of the samples was performed as described in Experimental procedures. Incubation times were (1), zero incubation; (2), 1.5 h; (3), 2 h; (4), 3 h; (5), 4 h.

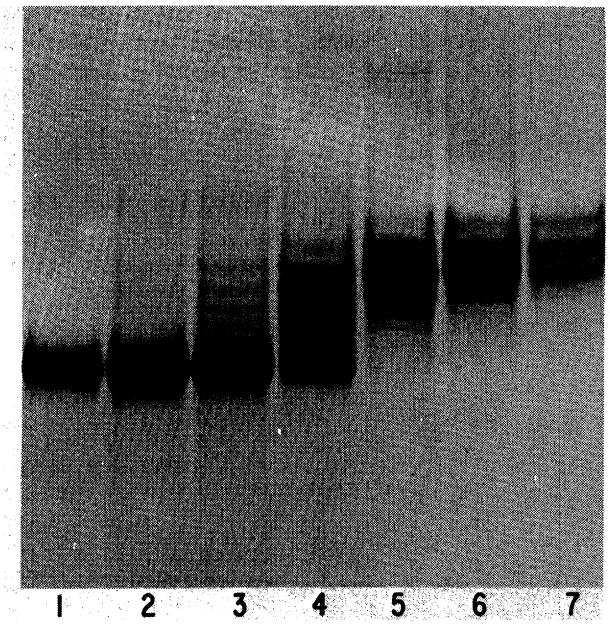


**Fig. 5. Polyacrylamide gel electrophoresis showing the effect of potato acid phosphatase concentration on $\alpha_{s1}$-casein. Varying amounts of potato acid phosphatase were added to 1-ml reaction mixtures containing 2.5 mg $\alpha_{s1}$-casein (pH 7.0). After incubating the samples for 2 h at 38°C, the solutions were frozen, freeze-dried, and electrophoresed (Experimental procedures). The amount of acid phosphatase added to each sample was (1), no acid phosphatase; (2), 0.1 μg; (3), 0.2 μg; (4), 0.5 μg; (5), 1.0 μg; (6), 2.0 μg; (7), 4.0 μg.**

*Enzyme concentration*

The effect of potato acid phosphatase concentration on the dephosphorylation of $\alpha_{s1}$-casein is shown in Fig. 5. Increasing the amount of enzyme in the reaction mixture increases the extent of dephosphorylation; the electrophoretic patterns show multiple bands with slower mobilities than the native $\alpha_{s1}$-casein. However, additional enzyme above 2 μg does not lead to complete removal of the phosphate groups of $\alpha_{s1}$-casein under the conditions used.

*Effect of dialysis*

Since potato acid phosphatase is inhibited by orthophosphate (Fig. 3), $\alpha_{s1}$-casein, following partial dephosphorylation, was dialyzed for 65 h at 4°C against distilled water to remove the phosphate ions. Following dialysis, $\alpha_{s1}$-casein migrated as a single band on polyacrylamide gel electrophoresis with a slower mobility than the partially dephosphorylated and untreated $\alpha_{s1}$-caseins (Fig. 6). The homogeneity of the dialyzed $\alpha_{s1}$-casein indicates that all the phosphate groups had been removed. A second incubation at 38°C was not needed since the potato acid phosphatase had sufficient activity at 4°C to remove the remaining phosphate groups during the dialysis.

*Preparation of dephosphorylated $\alpha_{s1}$-casein*

The following procedure was used to prepare large amounts of dephosphor-

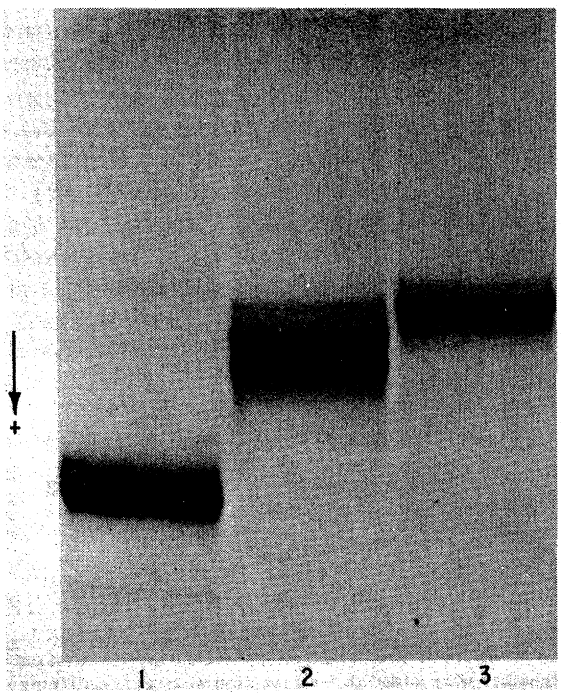


Fig. 6. Polyacrylamide gel electrophoresis showing the effect of dialysis following incubation of $\alpha_{s1}$-casein with potato acid phosphatase. The samples are (1), $\alpha_{s1}$-casein; (2), $\alpha_{s1}$-casein (25 mg) and 20 μg acid phosphatase in a 10-ml reaction mixture (pH 7.0) incubated for 2 h at 38°C; and (3), dialysis of sample 2 for 65 h at 4°C. Electrophoresis was performed on freeze-dried samples as described in Experimental procedures.

ylated casein. A 500-mg sample of $\alpha_{s1}$-casein was dissolved in 200 ml water and adjusted to pH 7.0. Following the addition of 0.4 mg potato acid phosphatase, the solution was incubated at 38°C for 2 h. The mixture was then dialyzed against 10 l of distilled water for 65 h at 4°C and freeze-dried. At this point the casein was examined by electrophoresis to determine whether the phosphate groups had been completely removed. Occasionally, it was necessary to repeat the procedure to obtain complete dephosphorylation. The casein was then precipitated by the addition of 1 M $CaCl_2$ to a final concentration of 0.02 M $CaCl_2$. After standing for 20 min at 38°C, the solution was centrifuged at 4000 × *g* for 15 min; 87% of the acid phosphatase activity remained in the supernatant. The $\alpha_{s1}$-casein precipitate was dissolved in 1 mM EDTA, (pH 7.0) and dialyzed overnight at 4°C against 1 mM EDTA to remove the calcium. The casein-EDTA was then dialyzed against water at 4°C to remove the EDTA. Following dialysis, the solution was adjusted to pH 7.6 and freeze-dried.

*Amino acid and phosphorus analyses*

The amino acid composition and the phosphorus content of native and dephosphorylated $\alpha_{s1}$-caseins are shown in Table III. Whole residues, computed from the known sequence of $\alpha_{s1}$-casein B, are shown for comparison. The

TABLE III

COMPOSITION OF NATIVE AND DEPHOSPHORYLATED $\alpha_{s1}$-CASEIN B

| Amino acid | Theory * | Mol residue/23 600 × g protein ** | |
|---|---|---|---|
| | | $\alpha_{s1}$-Casein B | Dephosphorylated $\alpha_{s1}$-casein B |
| Lys | 14 | 14.6 | 14.5 |
| His | 5 | 4.7 | 4.5 |
| $NH_3$ | 22 | 25.5 | 23.4 |
| Arg | 6 | 6.1 | 6.1 |
| Asp | 15 | 15.7 | 15.4 |
| Thr | 5 | 5.4 | 5.4 |
| Ser | 16 | 14.0 | 14.7 |
| Glu | 39 | 40.3 | 39.6 |
| Pro | 17 | 17.1 | 16.8 |
| Gly | 9 | 9.0 | 9.0 |
| Ala | 9 | 9.1 | 8.7 |
| Cys | 0 | 0 | 0 |
| Val | 11 | 9.3 | 10.4 |
| Met | 5 | 5.2 | 5.5 |
| Ile | 11 | 10.2 | 9.9 |
| Leu | 17 | 17.8 | 17.1 |
| Tyr | 10 | 10.3 | 10.1 |
| Phe | 8 | 8.1 | 7.8 |
| Trp *** | 2 | — | — |
| P | 8 | 8.6 | 0.22 |

* Calculated from the sequence of Mercier et al. [27].

** Analyses of 24 h hydrolysate using molar ratios based on Gly = 9.

*** Tryptophane was not determined.

amino acid composition of $\alpha_{s1}$-casein is identical to that of the dephosphorylated $\alpha_{s1}$-casein, providing strong evidence that the amino acids in $\alpha_{s1}$-casein were unaltered by potato acid phosphatase or contaminating enzymes. In addition, $\alpha_{s1}$-casein exposed to potato acid phosphatase for 4 h without dialysis showed no discernable peptides on high-voltage electrophoresis, indicating that no proteolysis had occurred. The major difference between the two proteins is their phosphorus content. Results in Table III show that potato acid phosphatase removed 97% of the phosphate groups of $\alpha_{s1}$-casein under the conditions described above.

*Effect of potato acid phosphatase on a variety of phosphoproteins*

Since potato acid phosphatase was effective in removing the phosphate groups from $\alpha_{s1}$-casein, the action of the enzyme was tested on a variety of phosphoprotein substrates under the conditions used for $\alpha_{s1}$-casein. Table IV shows that riboflavin binding protein, pepsinogen, $\alpha_{s1}$- and $\beta$-casein have less than 0.5 mol of phosphate/mol of protein, following treatment with potato acid phosphatase. Thus, these proteins were effectively dephosphorylated. Gel electrophoresis (Fig. 7) also shows that these proteins have been dephosphorylated. The dephosphorylated proteins are homogeneous on electrophoresis and have a slower mobility than the native proteins. The removal of phosphate groups decreases the net negative charge, causing the electrophoretic mobility on alkaline gels to decrease.

TABLE IV

REMOVAL OF PHOSPHATE GROUPS FROM VARIOUS PHOSPHOPROTEINS BY POTATO ACID PHOSPHATASE

Each phosphoprotein was treated with potato acid phosphatase in a 20-ml reaction mixture containing 50 mg of phosphoprotein and 40 μg enzyme at pH 7.0 for 2 h at 38°C. The solutions were then dialyzed against distilled water for 65 h at 4°C and freeze-dried. The native and dephosphorylated proteins were analyzed for protein and phosphorus as described under Experimental procedures.

| Protein | Molecular weight of native protein | Mol of phosphate residue/mol of protein * | | |
|---|---|---|---|---|
| | | Theory | Native protein | Dephosphorylated protein |
| $\alpha_{s1}$-Casein | 23 600 [27] | 8 [27] | 8.6 | 0.22 |
| β-Casein | 24 500 [28] | 5 [28] | 4.9 | 0.43 |
| Pepsinogen | 41 000 [23] | 1 [29] | 0.64 | 0.20 |
| Riboflavin binding protein | 32 000 [30] | 7 [30] | 6.6 | 0.33 |
| Ovalbumin | 45 000 [31] | 2 [31] | 1.7 | 0.79 |
| Phosvitin | 35 500 [31] | 113 [31] | 87.8 | 16.3 |

* The molecular weights of the dephosphorylated proteins were computed by subtracting the weight of the bound phosphate from the molecular weight of the native protein.

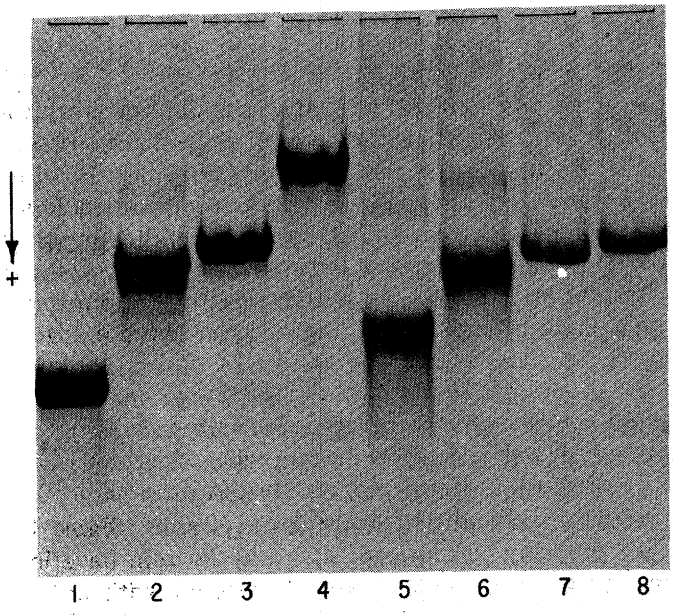


Fig. 7. Polyacrylamide gel electrophoresis of native and dephosphorylated proteins. The reaction mixture contained 50 mg phosphoprotein and 40 μg potato acid phosphatase in a 20-ml reaction mixture at pH 7. Following incubation for 2 h at 38°C, the solutions were dialyzed for 65 h at 4°C. The proteins were freeze-dried. The dephosphorylated and native proteins were subjected to gel electrophoresis (Experimental procedures). The samples are (1), $\alpha_{s1}$-casein; (2), dephosphorylated $\alpha_{s1}$-casein; 3, β-casein; 4, dephosphorylated β-casein; 5, riboflavin binding protein; 6, dephosphorylated riboflavin binding protein; 7, pepsinogen; 8, dephosphorylated pepsinogen.

Results indicate that only one of the two phosphate groups of ovalbumin was removed. Although 80% of the phosphate groups were removed from phosvitin, 16 mol remain (Table IV). The large number of phosphate groups on phosvitin suggests that the conditions used to dephosphorylate $\alpha_{s1}$-casein are not adequate for the complete dephosphorylation of phosvitin. It might be necessary to treat the phosvitin a second time with potato acid phosphatase to complete the reaction.

## Discussion

A variety of phosphatases have been used to remove the phosphate groups from phosphoproteins, such as casein, phosvitin, and pepsin. Enzymes that have been used with varying degrees of success are citrus fruit enzymes [32, 33], almond phosphatase [34], and spleen phosphoprotein phosphatase [1,2]. However, all these enzymes have been used at pH values below pH 6.0, which is not satisfactory for casein; $\alpha_{s1}$-casein is insoluble between pH 4.0 and pH 5.5 [38], while dephosphorylated $\alpha_{s1}$-casein in insoluble at pH 6.0 [1]. By using potato acid phosphatase at pH 7.0, problems due to the insolubility of casein are avoided. Another advantage of potato acid phosphatase is that it requires no activators or cofactors, while spleen phosphoprotein phosphatase requires reducing agents for maximum activity.

The results indicate that potato acid phosphatase is an effective phosphoprotein phosphatase. Although the *p*-nitrophenyl phosphatase activity is eight times higher than the phosphoprotein phosphatase activity when both are assayed at their pH optima, similar differences have been observed with spleen phosphoprotein phosphatase [35,36] and citrus fruit phosphatase [32]. In terms of its substrate specificity, potato acid phosphatase seems to resemble citrus fruit phosphatase, since both enzymes are active on phosphoserine as well as phosphoproteins and *p*-nitrophenyl phosphate. On the other hand, spleen phosphoprotein phosphatase differs from potato acid phosphatase in its substrate specificity. Although both enzymes can hydrolyze phosphoproteins and *p*-nitrophenyl phosphate, the spleen enzyme is unique because it acts on ATP, but not on phosphoserine [32,33].

Like most phosphatases, potato acid phosphatase is inhibited by phosphate ions (Fig. 3). The casein concentration used in these experiments was 0.11 mM or 0.85 mM in phosphate equivalents. The $K_i$ for phosphate, using *p*-nitrophenyl phosphate as a substrate, is 0.42 mM (Table I). Thus, it is essential to remove the phosphate ions in order to dephosphorylate the casein completely. The effect of phosphate inhibition should be even more pronounced in proteins, such as phosvitin, where the degree of phosphorylation is much higher than that of casein. Our experiments showed that under the conditions used to dephosphorylate a $\alpha_{s1}$-casein, only 80% of the phosphate groups of phosvitin were removed. Clark [34] digested phosvitin and a phosphopeptide of phosvitin with almond phosphatase and found that 20% of the original phosphoprotein phosphate remained as an unhydrolyzed core. He used two dephosphorylation steps with a dialysis between the two steps. We did not test the effect of a second treatment of phosvitin with potato acid phosphatase.

The preparation of homogeneous dephosphorylated $\alpha_{s1}$-casein was prompted

by our need to obtain dephosphorylated casein for use as a substrate for casein kinase [10] and this objective has been achieved. Since the presence of phosphoserine and phosphothreonine leads to complications in the sequence analyses of phosphoproteins and phosphopeptides, removal of phosphate residues by this method would also facilitate these investigations. The procedure described is simple and works well with a variety of phosphoproteins but it would have to be modified for dialyzable phosphopeptides.

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